Pharmacokinetics of 5 (and 6)-Carboxy-2′,7′-Dichlorofluorescein and Its Diacetate Promoiety in the Liver

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ABSTRACT

Hepatic disposition of 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF) and its diacetate promoiety (CDFDA) was studied in isolated perfused rat livers. Livers from Wistar wild-type and multidrug resistance-associated protein (Mrp)2-deficient (TR−) rats were perfused with CDF in the presence or absence of probenecid. Probenecid decreased the recovery of CDF in bile ~4-fold in wild-type livers (65 ± 8% versus 15 ± 2% of dose over 2 h). In livers from TR− rats, CDF was not excreted into bile and probenecid decreased perfusate CDF concentrations in a concentration-dependent manner, in part due to inhibition of Mrp3. Plasma membrane vesicles from Mrp2- or Mrp3-transfected Sf9 cells were used to confirm that CDF is a substrate for Mrp2 and Mrp3; probenecid inhibited the transport of CDF by Mrp2 and Mrp3 in a concentration-dependent manner.

CDF uptake in collagen sandwich-cultured rat hepatocytes was temperature-dependent and saturable (Km = 22 ± 10 μM; Vmax = 97 ± 9 pmol/min/mg protein). Uptake of CDF in sandwich-cultured rat hepatocytes was impaired significantly by bromosulfophthalein, a substrate for organic anion-transporting polypeptides (Oatps), but was not modulated by specific Oatp2 or organic anion transporter (Oat) substrates. CDFDA uptake was not saturable, temperature-dependent, or impaired by inhibitors. The hydrolysis of CDFDA to CDF is mediated by basic pH and esterases in biological media. CDFDA passively diffuses into hepatocytes where it is hydrolyzed to CDF. In contrast, CDF appears to be taken up by Oatp-mediated transport into rat hepatocytes and effluxed via Mrp2 into bile and via Mrp3 into sinusoidal blood.

The liver is one of the most important organs responsible for the detoxification and elimination of xenobiotics. The key role that hepatobiliary transport systems play in the uptake and excretion of many xenobiotics and metabolites into and out of hepatocytes has been recognized only recently. Many of the major organic anion transport proteins have been localized and cloned, and some functional characterization of these transporters has been published (Konig et al., 1999; Kullak-Ublick et al., 2000; Kusuha and Sugiyama, 2002). However, many compounds are substrates for more than one transport system, and this adds to the complexity of studying hepatobiliary transport in the whole cell and intact organ.

Probe compounds have been used to study alterations in hepatobiliary transport systems and to investigate the transport properties of new agents (Courtois et al., 1999; Payen et al., 2000). Fluorescent compounds can be assayed easily and with high sensitivity, thus having advantages over nonfluorescent compounds. The fluorophore 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF) is a multivalent organic anion at physiological pH (Fig. 1). The plasma membrane of cells presents a diffusional barrier for CDF. 5 (and 6)-Carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) carries only one negative charge (Leonhardt et al., 1971) and is permeable to cells. CDFDA, the diacetate promoiety used traditionally for CDF delivery to cells, diffuses through plasma membranes and is hydrolyzed to CDF by intracellular esterases (Breeuwer et al., 1995).

CDF is not subject to hepatic metabolism and has been used as a substrate for organic anion transport. The efflux of CDF was impaired in unpolarized hepatocytes from Mrp2-deficient TR− rats (Jansen et al., 1985, 1987) and Dubin-Johnson-like golden lion tamarins (Schulman et al., 1993), which was consistent with the recognition of CDF as an Mrp2 substrate (Kitamura et al., 1990). CDF has been used as a model compound to evaluate the biliary excretion of organic anions in sandwich-cultured rat hepatocytes (Liu et al., 1999b). Fluorescein also has been used extensively to study

ABBRévIATIONS: CDF, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein; CDFDA, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein diacetate; Mrp, multidrug resistance-related protein; TR− rats, Mrp2-deficient rats; HBSS, Hanks’ balanced salt solution; GFP, green fluorescence protein; Oatp, organic anion-transporting polypeptide; Oat, organic anion transporter.
Mrp1 transport in a variety of cell types (Huai-Yun et al., 1998; Sun et al., 2001).

Although CDF is eliminated from hepatocytes primarily via biliary excretion and, hence, appears to be a promising probe to study biliary excretion of organic anions, the hepatic transport of CDF has not been characterized fully. Alterations in hepatic disposition of CDF have been attributed traditionally to changes in biliary excretion, ignoring other relevant transport mechanisms, namely, uptake and basolateral efflux, where important interactions are likely to occur. In the present study, a multi-experimental approach was employed to examine the mechanisms of hepatic uptake, basolateral efflux, and canalicular excretion of CDF.

**Materials and Methods**

**Reagents.** CDF and CDFDA were purchased from Molecular Probes (Eugene, OR). Probenecid, sodium taurocholate (>97% pure), digoxin, bromosulfophthalein, p-aminophenacetic acid, rifampicin, and ouabain were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium, Grace's insect cell medium, SF9 cells, and BAC-TO-BAC Baculovirus Expression System were purchased from Invitrogen (Carlsbad, CA). Rat-tail collagen (type I) was obtained from BD Biosciences (Bedford, MA). All other chemicals were of reagent grade or the highest purity available.

**Animals.** Male Wistar rats (275–300 g; Charles River Laboratories, Inc. (Raleigh, NC) or male Mrp2-deficient TR− rats bred in our animal facility (275–300 g; obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used as liver donors in isolated perfused liver studies and hepatocyte isolation. Retired male Wistar breeders (>400 g, Charles River Laboratories) were used as blood donors. Rats were maintained on a 12-h light/dark cycle with access to water and rodent chow ad libitum. Rats were allowed to acclimate for at least 5 days before experimentation. Anesthesia was induced with ketamine/xylazine (60/12 mg/kg i.p.). The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures.

**Isolated Perfused Rat Liver Studies.** Recirculating isolated perfused liver experiments were performed using standard techniques (Brouwer and Thurman, 1996). Livers were allowed to acclimate for ~10 min before infusion of CDF or CDFDA. Bile was collected continuously every 15 min. Perfusion samples (~0.75 ml), collected every 15 min, were centrifuged immediately, and the supernatant was used for analysis. Measures of liver viability included portal pressure (<15 cm of H2O) and initial bile flow (>0.8 and >0.2 µl/min/g liver for wild-type and TR− rat livers, respectively). Unless specified otherwise, taurocholate (15 mM in saline, 2 ml/h) was infused continuously into the reservoir to maintain bile flow. To investigate the effects of taurocholate on CDF disposition, a set of experiments was performed without taurocholate. In the probenecid inhibition studies, probenecid (a bolus of 25 µmol followed by continuous infusion at 15.5 µmol/h) was administered to maintain steady-state perfuse concentrations of probenecid at ~260 µM (Turner, 1996). In a separate set of experiments in TR− livers, probenecid was administered at a 10-fold higher dose. CDFDA or CDF (10 mM in dimethyl sulfoxide, 0.01 ml/min, 35 min) was infused into the reservoir. Dimethyl sulfoxide (<0.05%, v/v) did not affect liver viability as determined by bile flow and the release of lactate dehydrogenase from the liver.

**Hepatocyte Culture in a Collagen Sandwich Configuration.** Hepatocyte isolation and culture were performed using standard techniques (Liu et al., 1998). Cell viability was measured by staining with trypan blue and was >90%. Hepatocytes were seeded on 60-mm polystyrene dishes coated with gelled collagen at a density of ~3 million cells/dish. Hepatocytes were overlaid with gelled collagen 24 h later. Hepatocytes were cultured for 4 days before experimentation to allow the formation of canalicular networks between cells.

**Hepatocyte Uptake Studies.** All culture dishes were rinsed with 3 ml of Hanks’ balanced salt solution (HBSS; 37°C or 4°C) prior to experimentation. To determine the concentration dependence of CDF or CDFDA uptake, cells were incubated in HBSS containing various concentrations of CDF or CDFDA (1–1000 µM) at 37°C for 10 min; the effect of temperature on uptake was examined by comparing uptake at 4°C or 37°C. Cells used for uptake at 4°C were prechilled before the cells were incubated in ice-cold HBSS containing 10 µM CDF or CDFDA. For inhibition studies, cells were preincubated with 3 µl of HBSS containing either inhibitors or vehicle at 37°C for 10 min, followed by incubation with 3 µl of HBSS containing 10 µM CDF or CDFDA with inhibitors or vehicle. After the incubation, dishes were rinsed with ice-cold HBSS and cells were lysed with 2 ml of lysing buffer [0.5% (v/v) Triton X-100 in phosphate-buffered saline]. Incubations in blank dishes coated with collagen were used to correct for nonspecific binding. Uptake clearance was calculated in the linear range as follows:

\[
\text{Uptake clearance} = \frac{dX/dt}{C_0},
\]

where \(X\) is the amount of CDF taken up by cells, and \(C\) is the CDF concentration in incubation medium.

**Protein Binding Assay.** Binding of CDF and CDFDA to the plasma proteins in perfusate was assessed with Centrifree Micropartition Devices (Millipore Corp., Bedford, MA), following a 5-min incubation (37°C) of either CDF or CDFDA (5 and 50 µM) with the supernatant obtained after centrifugation of perfusate, in the presence of various concentrations of probenecid (0, 260 µM, or 4 mM). CDF binding to the device was negligible.

**CDFDA Hydrolysis.** The conversion rates of CDFDA to CDF in perfusate and buffer (pH = 7.4) were determined in vitro. The effect of probenecid (0, 260 µM or 4 mM) on the conversion of CDFDA to
CDF in perfusate was investigated after a 5-min incubation (37°C; pH 7.4). The hydrolysis of CDFDA (initial concentration of 35 μM) in male Wistar rat blood and hepatic cytosolic fractions, prepared by centrifugation of tissue homogenates at 9000 × g for 15 min, was determined in the presence or absence of probenecid (300 μM, pH 7.4; 37°C) over 4 h to determine the linear range for CDFDA hydrolysis. The reaction was stopped at designated times by protein precipitation with cold acetonitrile. To determine the concentration dependence of CDFDA hydrolysis in cytosol, initial hydrolysis rates were measured over a range of CDFDA concentrations (10 min; 2.5–500 μM; n = 4 per concentration).

Production of Recombinant Baculovirus. Recombinant pFASTBAC1 plasmids containing either rat Mrp2, Mrp3, or green fluorescence protein (GFP) encoding sequence were kindly provided by Dr. Yuichi Sugiyama (University of Tokyo, Tokyo, Japan). Recombinant baculovirus was generated with BAC-TO-BAC Baculovirus Expression System according to the manufacturer’s instructions. Recombinant baculovirus stocks (>10^9 plaque-forming units/ml) were stored at 4°C. The viral titer of each stock was determined by plaque assays.

Viral Infection of Sf9 Insect Cells and Preparation of Plasma Membrane. Sf9 insect cells were cultured in spinner flasks at 27°C with Grace’s insect cell medium supplemented with 5% fetal bovine serum, 3.33 g/l lactalbumin hydrolysate, 3.33 g/l yeastolate, and antibiotics/antimycotics. Log phase Sf9 cells (1.0–1.5 × 10^6 cells/ml) were infected with recombinant baculovirus at a multiplicity of infection between 3 and 5, and harvested 3 days later. Plasma membranes were prepared as previously described (Huang et al., 1998).

Plasma Membrane Vesicle Uptake Studies. Uptake of 10 μM CDF into plasma membrane vesicles over 5 min was assessed in the presence or absence of probenecid after determining that uptake was linear during this time period. Substrate uptake into plasma membrane vesicles was measured by a quick filtration technique (Xiong et al., 2000). Briefly, aliquots of membrane suspensions (20 μl; 20–40 μg of protein) were preincubated for 5 min at 37°C, and uptake was initiated by the addition of 80 μl of prewarmed incubation buffer (20 mM HEPES (pH 7.5) 100 mM potassium nitrate, 100 mM sucrose, 5 mM hemimagnesium gluconate, 0.5 mM hemicalcium gluconate, 10 mM phosphocreatine, 100 μM/ml creatine phosphokinase, 10 mM magnesium chloride, 2 mM ATP) containing the substrate and the inhibitor to the membrane suspensions. Membrane vesicle uptake was terminated by addition of 3.5 ml of ice-cold membrane suspension buffer (20 mM HEPES/Tris (pH 7.4) 250 mM sucrose, 0.2 mM magnesium chloride). Vesicle-associated substrate was separated from free substrate by rapid filtration through a 0.45-μm filter. Filters were rinsed twice with 3.5 ml of ice-cold membrane suspension buffer. Filters were washed in 2 ml of lysis buffer (phosphate-buffered saline containing 0.5% Triton X-100) for 20 min at room temperature. Nonspecific binding of substrates to the filter was determined in the absence of membrane vesicles.

Analytical Methods. CDF concentrations in bile, perfusate, hepatocyte lysate, and Sf9 cell plasma membrane vesicle lysate from filters were determined by spectrophotometry (λmax/λaux. 505/523 nm) at pH 7.4 with a PerkinElmer LS50B luminescence spectrophotometer (PerkinElmer Life Sciences, Boston, MA). Samples were diluted in phosphate-buffered saline before determination. Standard curves of CDF (0.5–100 nM) were prepared daily and were linear (r^2 > 0.99). Protein concentrations were determined via a BCA protein assay kit (Pierce Chemical, Rockford, IL). Standard curves with bovine serum albumin (0.2–2 mg/ml) were prepared daily and were linear (r^2 > 0.99).

Pharmacokinetic Modeling. A compartmental modeling approach was employed to describe the hepatobiliary disposition of CDF and CDFDA in the isolated perfused livers of wild-type and TR^- rats. Various models employing linear and nonlinear processes were fit to the data. The goodness of fit of each model was assessed by visual examination of the distribution of residuals, the condition number, and Akaike’s Information Criterion (Akahei, 1976). Differential equations based on the concentration of CDF in the perfusate and the amount of CDF appearing in bile per unit time were resolved simultaneously by nonlinear least-squares regression with a weighting scheme of VY and the Gauss-Newton (Levenberg and Hartley) minimization method (WinNonlin 3.1; Pharsight Corporation, Mountain View, CA). The two-compartment model that best described the CDF infusion data in wild-type livers (Fig. 2A) was fit to CDF perfusate concentration-time and CDF biliary excretion rate data. The equations generated based on the scheme presented in Fig. 2A were as follows:

\[ V_p \cdot \frac{dC_p}{dt} = k_0 - k_{12} \cdot C_p \cdot V_p + k_{21} \cdot X_L, \]

\[ C_p^0 = 0, \quad k_0 = 0 \text{ at } t \geq 35 \text{ min} \]

\[ \frac{dX_L}{dt} = k_{12} \cdot C_p \cdot V_p - k_{21} \cdot X_L - k_{20} \cdot X_L, \quad X_L^0 = 0 \]

To describe CDF disposition during CDFDA infusion, \( k_0 \) was multiplied by the efficiency (84%) of the instantaneous conversion of the promoiety to CDF in perfusate.

The model was modified to describe CDF disposition during CDFDA infusion in TR^- livers. CDF excretion into bile of TR^- livers was negligible; \( k_{21} \) was thus set equal to zero. The estimated efficiencies of CDFDA conversion to CDF in perfusate in control, low-dose probenecid, and high-dose probenecid livers were set at 84%, 83%, and 63%, respectively. The estimated efficiencies were calculated from the ratio of CDF recovery from CDFDA and CDF in perfusate at a given probenecid concentration (see Table 2). The impact on the ratio of intercompartmental rate constants (\( k_{32}/k_{12} \)) was assessed for wild-type and TR^- livers. The TR^- data set, essentially perfusate concentrations for kinetic purposes, was not amenable to reliable estimation of uptake and basolateral efflux rate constants. The model could converge at numerous minima in the sum of squared error, whenever efflux and uptake were at an appropriate ratio, and thus only this ratio is reported and discussed for the TR^- data set. WinNonlin also was used to generate \( K_m \) and \( V_{max} \) estimates for saturable kinetics of CDF uptake.

Statistical Analyses. The Student’s two-tailed t test was used to assess statistical significance. Where variances were significantly different between two groups, Wilcoxon’s rank sum test was used to assess statistical significance. The criterion for significance in all cases was \( p < 0.05 \), with the Bonferroni correction where appropriate. All data are presented as mean ± S.D. except for hepatocyte uptake clearances, where the data represent the mean ± (S.E.M.) of means from three different animal preparations (n = 5/animal).

Results

Isolated Perfused Liver Studies. The biliary excretion of CDF in isolated perfused livers from TR^- rats was negligible in comparison with wild-type rats (Fig. 3A, Table 1). Total CDF recovery and recovery in bile were significantly higher after infusion of CDF compared with infusion of CDFDA at the same molar dose. In isolated perfused livers from wild-type rats, probenecid significantly impaired CDF biliary excretion while increasing CDF perfusate concentrations after the infusion of CDFDA (Fig. 3, A and B). In isolated perfused livers from TR^- rats, probenecid decreased perfusate concentrations of CDF after CDFDA administration in a concentration-dependent manner (Fig. 3C). Despite lower bile flow, the absence of the taurocholate confluence with CDFDA had no effect on the recovery of CDF in bile and perfusate in livers from wild-type rats.
Protein Binding of CDF and CDFDA. Binding of CDF and CDFDA to plasma proteins in perfusate (20% rat blood) was modest. The unbound fractions of CDF were 83/11006 4% and 87/11006 16% at 5 and 50/11006 M CDF, respectively, and 82/11006 7% and 86/11006 12% at 5 and 50/11006 M CDFDA, respectively. The protein binding of neither CDF nor CDFDA was altered by the presence of 260/11006 M or 4 mM probenecid in vitro.

CDFDA Hydrolysis Studies. The in vitro recovery of CDF in perfusate, administered as CDF, was not decreased in the presence of probenecid. In contrast, the in vitro recovery of CDF (Table 2) from CDFDA in perfusate was reduced by the presence of probenecid and was lower than that associated with free CDF at the same molar dose. The base hydrolysis of CDFDA to CDF was a first-order process with a half-life of 7.6/11006 0.1 h in phosphate-buffered saline at physiological pH and temperature (data not shown). In perfusate (20% rat blood), the rate of CDFDA hydrolysis to CDF was faster than detection permitted. Within 10 s after the addition of CDFDA to perfusate, the rate of CDFDA hydrolysis to CDF was faster than detection permitted. Within 10 s after the addition of CDFDA to perfusate, the rate of CDFDA hydrolysis to CDF was faster than detection permitted. 

Sf9 Cell Plasma Membrane Vesicle Uptake Studies. The uptake of CDF into GFP-expressing Sf9 cell plasma membrane vesicles in the presence of AMP or ATP was minimal. In contrast, CDF uptake into rat Mrp2- or Mrp3-expressing Sf9 cell plasma membrane vesicles was significantly higher in the presence of ATP than in the presence of AMP (Fig. 4). Probenecid inhibited CDF uptake into both Mrp2- and Mrp3-expressing Sf9 cell plasma membrane vesicles in a concentration-dependent manner, and to a similar extent (Fig. 5).

Hepatocyte Uptake Studies. The uptake clearance of CDFDA in rat hepatocytes cultured in a collagen sandwich configuration for 4 days was significantly higher than that of CDF (4.9/11006 0.3 versus 1.9/11006 0.1 l/min/mg protein; 10/11006 M). Low temperature (4°C) significantly decreased (2-fold) CDF uptake clearance but had no effect on CDFDA uptake (Fig. 6A). Uptake of CDFDA was a first-order process (Fig. 7B). Uptake of CDFDA at a concentration of 1 mM was assessed but is not reported due to very high binding to hepatocytes. CDF uptake was significantly inhibited by bromosulfophthalein (100/11006 M), but not by digoxin (100/11006 M) or p-aminohippurate (100 or 1000/11006 M) (Fig. 6B). Bromosulfophthalein, in combination with digoxin, did not inhibit CDF uptake further. Nonspecific Oatp inhibitors, taurocholate (100/11006 M), rifampicin (100/11006 M), ouabain (100/11006 M), and probenecid (260/11006 M), impaired the uptake of CDF by 41/11006 18, 32/11006 12, 38/11006 7, and 65/11006 16% of control (mean ± S.E.M.), respectively. In contrast to CDF, hepatic uptake of CDFDA was not inhibited by bromosulfophthalein, digoxin, or p-aminohippurate (data not shown).
Pharmacokinetic Modeling. Representative fits of the two-compartment model described in Fig. 2A to perfusate concentration and biliary excretion rate data are shown in Fig. 2B. First-order rate constants estimated for wild-type isolated perfused livers in the presence and absence of probenecid are summarized in Table 3. Probencicid significantly decreased the uptake and biliary excretion rate constants. Table 4 reports the ratios of basolateral efflux to uptake rate constants in wild-type and TR− livers. In wild-type livers, probencicid significantly increased this ratio, primarily due to the decrease in basolateral uptake. In TR− livers, probencicid decreased the basolateral efflux-to-uptake ratio in a concentration-dependent manner. Comparison of controls between the two groups indicated that TR− livers exhibited a significantly elevated ratio of basolateral efflux to uptake compared with livers from wild-type rats.

Discussion

The negligible biliary excretion of CDF in isolated perfused livers from Mrp2-deficient TR− rats compared with Wistar control rats was consistent with a previous report (Kitamura et al., 1990), suggesting Mrp2-mediated excretion of CDF in hepatocytes. Probencicid inhibited the excretion of CDF into bile. The concentration-dependent decrease in perfusate CDF concentrations by probenecid in isolated perfused rat livers from TR− rats could be explained by 1) inhibition of a basolateral efflux mechanism, or 2) a decrease in CDF available from the hydrolysis of CDFDA in the presence of probenecid. This second perturbation was incorporated in the pharmacokinetic modeling, so that only transport perturbations were estimated. The decreased perfusate concentrations of CDF in perfused livers from TR− rats can be attributed in part to probenecid inhibition of basolateral efflux of CDF by Mrp3, a major efflux route for anionic xenobiotics in the absence of Mrp2 (Ortiz et al., 1999; Ogawa et al., 2000).

Uptake studies with plasma membrane vesicles prepared from rat Mrp2- or Mrp3-expressing S9 cells demonstrated that CDF is a substrate for both Mrp2 and Mrp3. Probencicid inhibited the transport of CDF by Mrp2 and Mrp3 in a concentration-dependent manner. Despite a similar extent of Mrp2 and Mrp3 inhibition by probenecid, the inhibition of basolateral efflux may not be as important as that of biliary excretion because the biliary excretion is the dominant pathway for CDF efflux out of wild-type hepatocytes.

Collagen sandwich-cultured hepatocytes were used for uptake experiments because this culturing technique re-establishes canalicular networks and cell polarity (Liu et al., 1999a,c). Based on hepatocyte uptake studies, CDFDA appears to be taken up by a passive process, whereas CDF uptake is Oatp-mediated. CDFDA uptake was neither satu-

TABLE 1
Percentage of the dose recovered as CDF at the end of the 2-h perfusion Data are expressed as percentage of CDF or CDFDA dose recovered as CDF at the end of the 2-h perfusion in isolated perfused livers from wild-type and TR− rats. Values are mean ± S.D., n = 3/group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bile</th>
<th>Perfusate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type rats</td>
<td>CDFDA control</td>
<td>65 ± 8</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>CDFDA without taurocholate</td>
<td>65 ± 1</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>CDFDA + probenecid (260 μM)</td>
<td>15 ± 2*</td>
<td>48 ± 3*</td>
</tr>
<tr>
<td></td>
<td>CDFDA + probenecid (4 mM)</td>
<td>80 ± 5*</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>TR− rats</td>
<td>CDFDA control</td>
<td>0.05 ± 0.02</td>
<td>63 ± 2</td>
</tr>
<tr>
<td></td>
<td>CDFDA + probenecid (260 μM)</td>
<td>0.04 ± 0.01</td>
<td>54 ± 7</td>
</tr>
<tr>
<td></td>
<td>CDFDA + probenecid (4 mM)</td>
<td>0.04 ± 0.01</td>
<td>44 ± 5*</td>
</tr>
</tbody>
</table>

* p < 0.05, values compared with control in relevant rat strain.

TABLE 2
Recovery of CDF in perfusate Data are expressed as percentage of dose recovered as CDF at the end of the 5-min incubation at physiological temperature and pH. Data reflect CDF recovery from perfusate after CDF or CDFDA dose. Values are mean ± S.D., n = 3/group.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CDF 5 μM</th>
<th>50 μM</th>
<th>CDFDA 5 μM</th>
<th>50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91 ± 4</td>
<td>92 ± 2</td>
<td>81 ± 2*</td>
<td>72 ± 4*</td>
</tr>
<tr>
<td>Probenecid (260 μM)</td>
<td>93 ± 2</td>
<td>88 ± 2</td>
<td>83 ± 4</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>Probenecid (4 mM)</td>
<td>91 ± 6</td>
<td>90 ± 6</td>
<td>65 ± 3b</td>
<td>48 ± 2b</td>
</tr>
</tbody>
</table>

* p < 0.05, comparison of CDFDA to CDF controls.

b p < 0.05, values compared with control in relevant group.
rable nor temperature-dependent; in contrast, CDF uptake was significantly inhibited at 4°C and was saturable. CDF uptake does not appear to be Oat-mediated, because CDF uptake was not inhibited by p-aminohippurate. Recently, fluorescein accumulation in choroid plexus tissue was shown to be inhibited ~3-fold in the presence of 100 μM p-aminohippurate (Breen et al., 2002). Interestingly, CDF uptake was not inhibited even by 1 mM p-aminohippurate, suggesting that addition of the carboxyl group and two chlorines to the fluorescein molecule changes its substrate specificity. Uptake of CDF was digoxin-independent, suggesting that CDF may not be an Oatp2 substrate. However, recently, Meng et al. (2002) demonstrated that digoxin did not inhibit the uptake of an Oatp2 substrate, sulfobiltrihyltaurine. CDF appears to be an Oatp1 substrate based on bromosulfophthalein inhibition of CDF uptake. Since bromosulfophthalein is very highly protein-bound (Reyes et al., 1969), to ensure that the decrease in intracellular accumulation of CDF was due to inhibition of transport by Oatp1 and not a protein binding effect, the results of the study were confirmed with nonspecific Oatp substrates. Probencid, a nonspecific organic anion transport inhibitor (Sugiyama et al., 2001), also inhibited the uptake of CDF. Recently, rat Oatp4 has been shown to mediate hepatic uptake of xenobiotics (Cattori et al., 2001). Identification of the specific Oatp isoforms responsible for the hepatic uptake of CDF requires further investigation. Uptake of CDFDA was not inhibited by bromosulfaphthalein, digoxin, p-aminohippurate, or probencid, which further confirms that uptake of the promoiety is a passive process.

Pharmacokinetic analysis yielded further insight into CDF transport. Fitting the two-compartment model to the data from isolated perfused livers from wild-type rats in the presence or absence of probenecid elucidated the sites of inhibition. Uptake and biliary excretion were inhibited significantly (10- and 2-fold, respectively) by probenecid in wild-type livers. The two sites of inhibition indicate that both hepatic uptake and biliary excretion must be considered in examining mechanisms of inhibition, especially in cases where uptake is the rate-limiting step in hepatic elimination. Traditionally, interactions at the site of biliary excretion have been emphasized. Interestingly, probenecid coadministration did not decrease the basolateral efflux rate constant despite the ability of probenecid to inhibit Mrp3 in vitro. The absence of the anticipated decrease in this rate constant may be due to increased intrahepatic CDF concentrations second-

Fig. 4. Uptake of CDF by plasma membrane vesicles from GFP-transfected (◼, □) SF9 cells or rat Mrp2-transfected (○, ●) SF9 cells in the presence of AMP (open symbols) or ATP (solid symbols) (A). Corresponding uptake of CDF by plasma membrane vesicles from GFP- or Mrp3-transfected SF9 cells (B). Values are mean ± S.D.; n = 3/group.

Fig. 5. Relative uptake of CDF in rat Mrp2-transfected (A) and Mrp3-transfected (B) SF9 cell plasma membrane vesicles in the absence or presence of probenecid (PRB). Data are normalized for nonspecific CDF uptake by the vesicles. Values are mean ± S.D.; n = 3/group; *, p < 0.05, probenecid versus control.
ary to a decrease in biliary excretion. Clearly, basolateral efflux plays an important role when biliary excretion is compromised. In fact, in livers from TR/H11002 rats in the absence of probenecid, where biliary excretion is negligible due to the absence of Mrp2, the ratio of basolateral efflux to uptake was significantly increased 100-fold (15.3 ± 6.8 versus 0.13 ± 0.17), just as when probenecid was administered to isolated perfused livers from wild-type rats where the ratio increased 30-fold (3.4 ± 1.6 versus 0.13 ± 0.17) relative to wild-type controls. In TR/H11002 rats, probenecid decreased the basolateral efflux-to-uptake ratio in a concentration-dependent manner. Pharmacokinetic modeling suggests that when basolateral efflux is the only route for CDF elimination, basolateral transport proteins such as Mrp3 may be inhibited to a greater extent than Oatp-mediated uptake. Mrp3 expression in TR/H11002 rats is much greater than in wild-type rats (Xiong et al., 2008).

**TABLE 3**

Parameter estimates for CDF disposition in livers from wild-type rats in the presence or absence of probenecid.

<table>
<thead>
<tr>
<th>Rate constant (min⁻¹)</th>
<th>Control</th>
<th>Probenecid (260 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁₂</td>
<td>0.024 ± 0.005</td>
<td>0.0023 ± 0.0006</td>
</tr>
<tr>
<td>k₂₁</td>
<td>0.003 ± 0.004</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>k₃₀</td>
<td>0.16 ± 0.05</td>
<td>0.083 ± 0.006*</td>
</tr>
</tbody>
</table>

* p < 0.05, values compared with control.

**TABLE 4**

Ratio of intercompartmental rate constants (basolateral efflux/uptake) for CDF disposition in livers from wild type and Mrp2-deficient rats in the presence or absence of probenecid.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio in Wild-Type</th>
<th>Ratio in TR Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDF control</td>
<td>0.13 ± 0.17</td>
<td>15.3 ± 6.8*</td>
</tr>
<tr>
<td>CDF + probenecid (260 μM)</td>
<td>3.4 ± 1.6b</td>
<td>5.5 ± 3.3b</td>
</tr>
<tr>
<td>CDF + probenecid (4 mM)</td>
<td>N/A</td>
<td>2.4 ± 0.7b</td>
</tr>
</tbody>
</table>

* p < 0.05, comparison of mutant to wild-type.  
  b p < 0.05, values compared with control in relevant group.  
  N/A not assessed.

**Fig. 6.** Effect of temperature and inhibitors on the uptake clearance of CDF and CDFDA in day 4 sandwich-cultured rat hepatocytes. The uptake clearance of CDF (10 μM) and CDFDA (10 μM) was evaluated at 10 min at 4°C and 37°C (A). The uptake clearance of CDF (10 μM, 10 min) was determined in the presence of inhibitors (preincubated for 10 min at 37°C) for Oatp1, bromosulfophthalein (BSP); Oatp2, digoxin (Dig); Oat2, 100 μM p-aminohippurate (PAH); and Oat3, 1 mM p-aminohippurate (B). Values are mean ± (S.E.M.) of means from three different animal preparations (n = 5/animal); * p < 0.05 inhibitor versus control.

**Fig. 7.** Uptake rate of CDF over the 1 to 1000 μM range (A) and uptake rate of CDFDA over the 1 to 500 μM range (B). Lines represent the fit of a saturable (A) and linear (B) uptake model to the data. Values are mean ± (S.E.M.) of means from three different animal preparations (n = 5/animal).

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Hepatic Disposition of Carboxydichlorofluorescein 807 at ASPET Journals on September 6, 2017 jpet.aspetjournals.org Downloaded from anet.aspetjournals.org at ASPET Journal on September 6, 2017
The present study demonstrated that probenecid could inhibit human MRP1 organic anion transport. However, sites and mechanisms of rate.

CDFDA in buffer at physiological temperature and pH is not used (1–10 μM). Attempting CDFDA delivery in a biological matrix, such as perfusate of the isolated perfused liver, extracellular media by basic conditions or esterases to CDF. CDF is taken up via an Oatp-mediated process and is excreted from the liver by Mrp2 on the basolateral membrane. CDFDA uptake at the dosing concentrations most commonly used (1–10 μM) could be mediated by several transporters, and to fully understand the hepatic transport of a compound, all processes, not merely excretion into bile, must be considered.

In summary, hepatic transport of CDF has been characterized (Fig. 8). CDF is taken up into hepatocytes by a saturable, temperature-dependent Oatp-mediated mechanism. In contrast, the diacete promoiety, CDFDA, is taken up by passive diffusion and instantaneously hydrolyzed by intracellular esterases to CDF. The disadvantage of CDFDA is its instability in biological media. CDF is excreted from liver into bile by Mrp2 and from liver into sinusoidal blood by Mrp3. When Mrp2-mediated excretion of CDF is impaired, basolateral efflux by Mrp3 increases.

Acknowledgments
We thank Dr. Yuichi Sugiyama for rat Mrp2, Mrp3, and green fluorescence protein cDNA. The insightful advice of Dr. Pieter Annaert is greatly appreciated.

References

Fig. 8. Proposed scheme for hepatic transport of CDF and CDFDA. CDFDA is either passively taken up by the liver, where it is instantaneously cleaved by esterases, or is hydrolyzed in the extracellular medium by basic conditions or esterases to CDF. CDF is taken up via an Oatp-mediated process and is excreted from the liver by Mrp2 on the canalicular membrane and Mrp3 on the basolateral membrane.

SINUSOIDAL BLOOD

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